MAIL TO APPLICANT

16 45

PCT WORLD INTELLECTUAL PROPERTY International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER TH

WO 9606190A2

(51) International Patent Classification 6 : C12Q 1/68, C12P 19/34, C07H 21/04 — A2

(11) International Publication Number: WO 96/06190

(43) International Publication Date: 29 February 1996 (29.02.96)

(21) International Application Number: PCT/US95/10603

(22) International Filing Date: 17 August 1995 (17.08.95)

(30) Priority Data:
08/292,686 19 August 1994 (19.08.94) US

(71) Applicant: PERKIN-ELMER CORPORATION [US/US]; Applied Biosystems Division, 850 Lincoln Centre Drive, Foster City, CA 94404 (US).

(72) Inventor: EGGERDING, Faye; Apartment 5L, 8 Locksley, San Francisco, CA 94122 (US).

(74) Agents: WEITZ, David, J. et al.; Haynes & Davis, Suite 310, 2180 Sand Hill Road, Menlo Park, CA 94025-6935 (US).

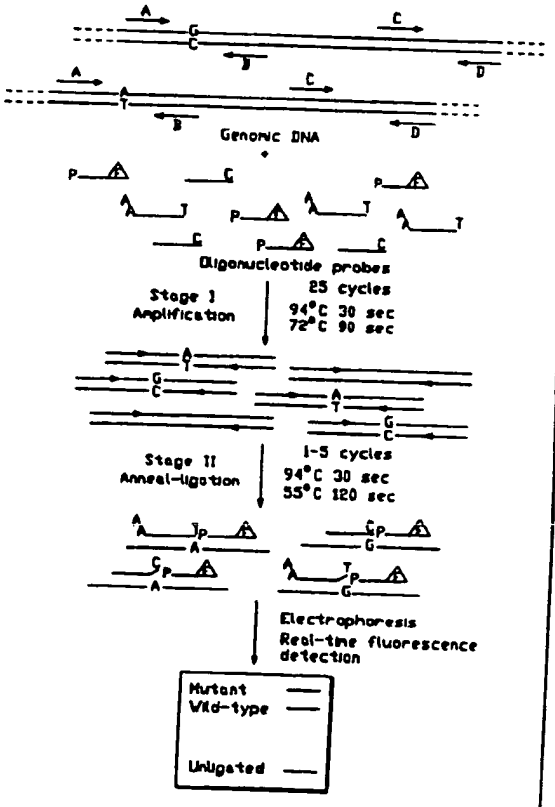
(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: COUPLED AMPLIFICATION AND LIGATION METHOD

(57) Abstract

A method based on polymerase chain reaction (PCR) amplification and oligonucleotide ligase assay (OLA) reaction is provided for analyzing complex genetic systems in a single reaction vessel. The method involves simultaneously incubating a sample containing one or more target polynucleotides with PCR primers and OLA probes in a single reaction mixture. The presence of variant polynucleotide sequences in the sample is determined by detecting and identifying the products of the OLA reaction.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

COUPLED AMPLIFICATION AND LIGATION METHOD

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to methods of detecting and/or distinguishing known DNA sequence variants. More specifically, the invention pertains to a method of performing DNA amplification reactions and oligonucleotide ligase assay reactions in a single reaction vessel with minimal post-amplification sample manipulation to detect and/or distinguish known DNA sequence variants.

Description of Related Art

Nucleic acid sequence analysis has become important in many research, medical, and industrial fields, e.g. Caskey, Science 236: 1223-1228 (1987); Landegren et al, Science, 242: 229-237 (1988); and Arnheim et al, Ann. Rev. Biochem., 61: 131-156 (1992). In large part, the strong interest in nucleic acid analysis has been driven by the development of several methods for amplifying target nucleic acids, e.g. polymerase chain reaction (PCR), ligation chain reaction (LCR), and the like, e.g. Kessler, editor, Nonradioactive Labeling and Detection of Biomolecules (Springer-Verlag, Berlin, 1992); Innis et al, editors, PCR Protocols (Academic Press, New York, 1990); Barany, PCR Methods and Applications 1: 5-16 (1991).

While such amplification techniques have the potential of providing highly sensitive and specific diagnostic assays, there is still a need to make assays utilizing such techniques convenient to perform in a clinical or field setting, especially when they involve the analysis of complex genetic systems, such as the extremely variable cystic fibrosis locus, or other highly polymorphic loci. In such systems, identifying the amplified product poses a special problem whose solution typically requires multiple post-amplification manipulations. A promising approach for identifying polynucleotides in such systems is the oligonucleotide ligation assay (OLA), Whiteley et al, U.S. patent 4,883,750. In this assay approach, oligonucleotides are prepared that are complementary to adjacent regions of a target sequence. The oligonucleotides are capable of

hybridizing to the target so that they lie end-to-end and can be ligated when no mismatches occur at or near the contiguous ends. Whenever such mismatches do occur, then ligation is precluded. As a result, a set of oligonucleotide pairs may be provided which are perfect complements of all the allelic variants of interest at a given locus. By a judicious selection of labeling methodologies, a wide range of alleles, either from the same of different loci, can be specifically identified in a single assay.

Unfortunately, application of OLA to amplified target sequences complicates the assay, as exemplified by Nickerson et al., Proc. Natl. Acad. Sci. USA 87:8923-8927 (1990), which discloses the amplification of target DNAs by PCR and discrimination of variant DNA by OLA. After PCR amplification of the target DNA was performed in a first set of 96-well cluster plates, aliquots of the amplified samples were transferred to a second set of 96-well plates for OLA and the generation of ligation products. Aliquots of samples containing the ligation products were then transferred from the second set of plates to a third set of 96-well plates for detection of ligation products by an ELISA-based procedure.

The application of DNA-based assays employing amplification and OLA detection would be greatly facilitated if the number manipulations required to implement the assays could be reduced.

SUMMARY OF THE INVENTION

The present invention provides a method of amplifying and detecting by OLA in the same reaction vessel one or more target polynucleotides in a sample. An important aspect of the invention is providing primers, or oligonucleotides (in the case of ligation-based amplification), for target polynucleotide amplification having a higher annealing temperature than that of the oligonucleotides employed in the OLA. In this manner, the target polynucleotides is amplified at a temperature above the annealing temperature of the oligonucleotides employed in the OLA, thereby avoiding the interference to chain extension and/or ligation that would occur were the oligonucleotides allowed to anneal to the target polynucleotides during amplification.

Generally, the method of the invention comprises the steps of (a)

providing a plurality of amplification primers, each amplification primer being capable of annealing to one or more target polynucleotides at a first annealing temperature, (b) providing a plurality of oligonucleotide probes, each oligonucleotide probe of the plurality being capable of annealing to the target polynucleotides at a second annealing temperature, such that substantially none of the oligonucleotide probes anneal to the target polynucleotide at the first annealing temperature, (c) amplifying the target polynucleotides using the plurality of amplification primers at a temperature greater than or equal to the first annealing temperature; (d) ligating oligonucleotide probes of the plurality that specifically hybridize to the one or more target polynucleotides at a temperature equal to or less than the second annealing temperature to form one or more ligation products; and (e) detecting the one or more ligation products. The presence or absence of the ligation products is then correlated to the presence or absence of the one or more target polynucleotides in the sample.

The invention further includes kits for carrying out the method of the invention. Preferably, such kits include (a) a plurality of amplification primers, each amplification primer of the plurality being capable of annealing to one or more target polynucleotides at a first annealing temperature; (b) a plurality of oligonucleotide probes, each oligonucleotide probe being capable of annealing to the target polynucleotides at a second annealing temperature, such that substantially none of the oligonucleotide probes anneal to the target polynucleotide at the first annealing temperature; (c) means for amplifying the target polynucleotides using the plurality of amplification primers at a temperature greater than or equal to the first annealing temperature; and (d) means for ligating oligonucleotide probes at a temperature equal to or less than the second annealing temperature to form one or more ligation products.

The invention overcomes a deficiency attendent to current approaches by permitting the amplification and detection by OLA in a single reaction vessel, thereby reducing the amount of sample and reagent manipulations required in such an assay. The method of the invention is readily automated. Generally, the method can be used to assay, simultaneously, target sequences, such as sequences associated with a mixture of pathogen

specimens, gene sequences in a genomic DNA fragment mixture, highly polymorphic or mutationally complex genetic loci, such as the cystic fibrosis locus, p53 locus, ras locus, or the like.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of one embodiment of the invention.

Figure 2A and 2B are electropherograms of fluorescently labeled ligation products.

Definitions

As used in reference to the method of the invention, the term "target polynucleotide" in the plural includes both multiple separate polynucleotide strands and multiple regions on the same polynucleotide strand that are separately amplified and/or detected. A target polynucleotide may be a single molecule of double-stranded or single-stranded polynucleotide, such as a length of genomic DNA, cDNA or viral genome including RNA, or a mixture of polynucleotide fragments, such as genomic DNA fragments or a mixture of viral and somatic polynucleotide fragments from an infected sample. Typically, a target polynucleotide is double-stranded DNA which is denatured, e.g., by heating, to form single-stranded target molecules capable of hybridizing with primers and/or oligonucleotide probes.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, polyamide nucleic acids, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, and capable of being ligated to another oligonucleotide in a template-driven reaction. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3-4, to several hundreds of monomeric units. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'->3' order from left to right and that "A"

denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. The term "polynucleotide" as used herein usually means a linear oligomer of nucleosides or analogs thereof, including deoxyribonucleosides, ribonucleosides, and the like, from a few tens of units in length to many thousands of units in length.

As used herein, "pluality" in reference to oligonucleotide probes includes sets of two or more oligonucleotide probes where there may be a single "common" oligonucleotide probe that is usually specific for a non-variable region of a target polynucleotide and one or more "wild-type" and/or "mutant" oligonucleotide probes that are usually specific for a region of a target polynucleotide that contains allelic or mutational variants in sequence.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990); or the like. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce degeneracy, increase specificity, and the like.

The term "amplification primer", as used herein, refers to an oligonucleotide which either (i) acts to initiate synthesis of a complementary DNA strand when placed under conditions in which synthesis of a primer extension product is induced, i.e., in the presence of nucleotides and a polymerization-inducing agent such as a DNA-dependent DNA polymerase and at suitable temperature, pH, metal concentration, and salt concentration, or (ii) is ligated to another amplification primer in a ligation-based amplification scheme.

DETAILED DESCRIPTION OF THE INVENTION

The invention eliminates the need to provide separate reaction mixture and/or vessels for applying OLA to amplified target polynucleotides. In

accordance with the method, target polynucleotides are amplified above a first temperature (i.e., the first annealing temperature) in the presence of the oligonucleotide probes of the OLA. At or above this first temperature, the OLA components of the reaction mixture do not interfere with amplification. After amplification, the temperature of the reaction mixture is lowered to a second temperature (i.e., the second annealing temperature) that permits specific annealing of the oligonucleotide probes of the OLA to the target polynucleotides. The reaction mixture then may be cycled between the second temperature and a higher temperature to permit linear amplification of ligation products.

Preferably, amplification primers are from 30 to 50 nucleotide long and have Tm's between 80°C and 120°C. Preferably, such amplification primers are employed with a first annealing temperature of between about 72°C to about 84°C. More preferably, the first annealing temperature is between about 72°C to about 75°C. Preferably, the oligonucleotide probes used in the OLA are from 8 to 30 nucleotides long and have Tm's between 40°C and 70°C. Such oligonucletide probes are preferably used with a second annealing temperature between about 30°C to about 55°C, and more preferably, between about 40°C to about 55°C. Preferably, annealing temperatures are selected to ensure specificity in amplification and detection. Typically, annealing temperatures are selected in the range of from 1-2°C above or below the melting temperature of an amplification primer or oligonucleotide probe to about 5-10°C below such temperature. Guidance for selecting appropriate primers or oligonucleotides given these design constraints and the nature of the polynucleotide targets can be found in many references, including Rychlik et al. (1989) Nucl. Acids. Res. 17:8453-8551; Lowe et al. (1990) Nucl. Acids Res. 18:1757-1761; Hiller et al. (1991) PCR Methods and Applications 1:124-128; Wetmur, Critical Reviews in Biochemistry and Molecular Biology, 26: 227-259 (1991); Breslauer et al, Proc. Natl. Acad. Sci. 83: 3746-3750 (1986); Innis et al, editors, PCR Protocols (Academic Press, New York, 1990); and the like.

Amplification primers and oligonucleotide probes for OLA reactions are readily synthesized by standard techniques, e.g., solid phase synthesis via phosphoramidite chemistry, as disclosed in U.S. Patent Nos. 4,458,066 and

4,415,732 to Caruthers et al; Beaucage et al. (1992) Tetrahedron 48:2223-2311; and Applied Biosystems User Bulletin No. 13 (1 April 1987). Likewise, the primers and oligonucleotide probes are derivatized with reactive groups, e.g. for attaching labels, using conventional chemistries, such as disclosed in Eckstein, editor, Oligonucleotides and Analogues: A Practical Approach (IRL Press, Oxford, 1991).

Ligation products generated in the method are detected by a variety of means. For example, detection may be achieved by coupling a detectable label to the terminus of one of the oligonucleotide probes. Alternatively, the non-ligating termini of the oligonucletides may be labeled with distinct labels which are detectable by spectroscopic, photochemical, biochemical, immunochemical or radiochemical means. Detection may also be achieved by using a nucleic acid hybridization assay, e.g. as described in Urdea et al, U.S. patent 5,124,246; or like techniques.

Preferably, ligation products bear mobility modifiers. i.e., extensions which allow the mobility of each ligation product to be defined so that they may be distinguished by methods which provide size dependent separation, such as sedimentation, exclusion chromatography, filtration, high performance liquid chromatography, electrophoresis, affinity collection, or the like. Most preferably, such mobility modifiers alter the electrophoretic mobility of the ligation products rendering them separately detectable. Preferably, the wild type allele OLA products are separated from mutant allele OLA products by electrophoresis or capillary electrophoresis, particularly gel-free capillary electrophoresis. More preferably, amplified OLA products containing mobility modifiers are detectably labeled and separated by gel electrophoresis on an instrument such as a model 373 DNA Sequencer (Applied Biosystems, Foster City, CA), described in the following references: Mayrand et al. (1990) Clin. Chem. 36:2063-2071; and Mayrand et al. (1992) Appl. Theoret. Electrophoresis 3:1-11. Synthesizing and attaching mobility modifiers to oligonucleotides and their use in OLA is described in International applications PCT/US93/20236 and PCT/US93/20239, which are incorporated by reference.

As taught in these applications, a variety of mobility modifying elements are attached to oligonucleotide probes, including polymer chains formed of

polyethylene oxide, polyglycolic acid, polylactic acid, polypeptide, oligosaccharide, polyurethane, polyamids, polysulfonamide, polysulfoxide, and block copolymers thereof, including polymers composed of units of multiple subunits linked by charged or uncharged linking groups.

An important feature of this embodiment of the invention is the use of different mobility modifying polymer chains for imparting different ratios of charge/translational frictional drag to different ligation products. That is, the ratio of combined charge/combined translational frictional drag of the oligonucleotide, attached polymer chain, and label, as measured at a given pH and with respect to electrophoretic polymer movement through a non-sieving liquid medium, is different for each different-sequence ligation product. Preferably, the distinctive ratio of charge/translational frictional drag is typically achieved by differences in the lengths (number of subunits) of the polymer chain. However, differences in polymer chain charge are also contemplated, as are differences in oligonucleotide length.

More generally, the polymers forming the polymer chain may be homopolymers, random copolymers, or block copolymers, and the polymer may have a linear, comb, branched, or dendritic architecture. In addition, although the invention is described herein with respect to a single polymer chain attached to an associated binding polymer at a single point, the invention also contemplates binding polymers which are derivatized by more than one polymer chain element, where the elements collectively form the polymer chain.

Preferred polymer chains are those which are hydrophilic, or at least sufficiently hydrophilic when bound to the oligonucleotide binding polymer to ensure that the probe is readily soluble in aqueous medium. The polymer chain should also not effect the hybridization reaction. Where the binding polymers are highly charged, as in the case of oligonucleotides, the binding polymers are preferably uncharged or have a charge/subunit density which is substantially less than that of the binding polymer.

Methods of synthesizing selected-length polymer chains, either separately or as part of a single-probe solid-phase synthetic method, are described below, along with preferred properties of the polymer chains.

In one preferred embodiment, described below, the polymer chain is formed of hexaethylene oxide (HEO) units, where the HEO units are joined end-to-end to form an unbroken chain of ethylene oxide subunits, or are joined by charged or uncharged linkages, as described below.

Methods of preparing polymer chains in the probes generally follow known polymer subunit synthesis methods. These methods, which involve coupling of defined-size, multi-subunit polymer units to one another, either directly or through charged or uncharged linking groups, are generally applicable to a wide variety of polymers, such as polyethylene oxide, polyglycolic acid, polylactic acid, polyurethane polymers, and oligosaccharides.

The methods of polymer unit coupling are suitable for synthesizing selected-length copolymers, e.g., copolymers of polyethylene oxide units alternating with polypropylene units. Polypeptides of selected lengths and amino acid composition, either homopolymer or mixed polymer, can be synthesized by standard solid-phase methods. Preferably, PEO chains having a selected number of HEO units are prepared from DMT-protected phosphoramidite monomers, as disclosed in Levenson et al, U.S. patent 4,914,210.

Coupling of the polymer chains to an oligonucleotide can be carried out by an extension of conventional phosphoramidite oligonucleotide synthesis methods, or by other standard coupling methods. Alternatively, the polymer chain can be built up on an oligonucleotide (or other sequence-specific binding polymer) by stepwise addition of polymer-chain units to the oligonucleotide, using standard solid-phase synthesis methods.

As noted above, the polymer chain imparts to its probe, a ratio of charge/translational frictional drag which is distinctive for each different-sequence probe and/or ligation product. The contribution which the polymer chain makes to the derivatized binding polymer will in general depend on the subunit length of the polymer chain. However, addition of charge groups to the polymer chain, such as charged linking groups in the PEO chain, or charged amino acids in a polypeptide chain, can also be used to achieve selected charge/frictional drag characteristics in the probe.

An important feature of this embodiment of the invention is providing ligation products of different-length and/or different-sequence oligonucleotides which can be finely resolved electrophoretically in a non-sieving medium by derivatization with polymer chains having slightly different size and/or charge differences. Electrophoresis, such as capillary electrophoresis, (CE) is carried out by standard methods, and using conventional CE equipment.

The ability to fractionate charged binding polymers, such as oligonucleotides, by electrophoresis in the absence of a sieving matrix offers a number of advantages. One of these is the ability to fractionate charged polymers all having about the same size. This feature allows the oligonucleotide moiety of the probes to have similar sizes, and thus similar hybridization kinetics and thermodynamics with the target polynucleotide. Another advantage is the greater convenience of electrophoresis, particularly CE, where sieving polymers and particularly problems of forming and removing crosslinked gels in a capillary tube are avoided.

In the above OLA, the concentration of ligation product can be enhanced, if necessary, by repeated probe hybridization and ligation steps. Simple linear amplification can be achieved using the target polynucleotide as a template and repeating the denaturation, annealing, and probe ligation steps until a desired concentration of derivatized probe is reached.

In order to carry out the method of the invention, then, a sample is provided which includes DNA containing one or more target nucleotide sequences. Chromosomal DNA of an individual who is being tested or screened is obtained from a cell sample from that individual. Cell samples can be obtained from a variety of tissues depending on the age and condition of the individual. Preferably, cell samples are obtained from peripheral blood using well known techniques. In fetal testing, a sample is preferably obtained by amniocentesis or chorionic villi sampling. Other sources of DNA include semen, buccal cells, or the like. Preferably, DNA is extracted from the sample using standard procedures, e.g., phenol:chloroform extraction as described by Maniatis et al., supra, and Higuchi (May 1989) PCR Applications, Issue 2 (Perkin Elmer-Cetus Users Bulletin). Cell samples for fetal testing can also be

obtained from maternal peripheral blood using fluorescence-activated cell sorting, as described, e.g., by Iverson et al. (1981) Prenatal Diagnosis 9:31-48.

The method of the invention involves the specific amplification of target polynucleotides by PCR or ligation-based amplification to provide templates for the subsequent OLA. Ligation-based polynucleotide amplification, such as ligase chain reaction, is disclosed in the following references: Barany, PCR Methods and Applications 1: 5-16 (1991); Landegren et al, U.S. patent 4,988,617; Landegren et al, Science 241: 1077-1080 (1988); Backman et al, European patent publication 0439182A2; Yu and Wallace, Genomics 4: 560-569 (1989);and the like.

The PCR method for amplifying target polynucleotides in a sample is well known in the art and has been described by Saiki et al. (1986) Nature 324:163, as well as by Mullis in U.S. Patent No. 4,683,195, Mullis et al. in U.S. Patent No. 4,683,202, Gelfand et al. in U.S. Patent No. 4,889,818, Innis et al. (eds.) PCR Protocols (Academic Press, NY 1990), and Taylor (1991) Polymerase chain reaction: basic principles and automation, in PCR: A Practical Approach, McPherson et al. (eds.) IRL Press, Oxford.

Briefly, the PCR technique involves preparation of oligonucleotide primers which flank the target nucleotide sequence to be amplified, and are oriented such that their 3' ends face each other, each primer extending toward the other. The polynucleotide sample is extracted and denatured, preferably by heat, and hybridized with the primers which are present in molar excess. Polymerization is catalyzed in the presence of deoxyribonucleotide triphosphates (dNTPs) as noted above. This results in two "long products" which contain the respective primers at their 5' ends covalently linked to the newly synthesized complements of the original strands. The reaction mixture is then returned to polymerizing conditions, e.g., by lowering the temperature, inactivating a denaturing agent, or adding more polymerase, and a second cycle is initiated. The second cycle provides the two original strands, the two long products from the first cycle, two new long products replicated from the original strands, and two "short products" replicated from the long products. The short products have the sequence of the target sequence with a primer at each end. On each additional cycle, an additional two long products are

produced, and a number of short products equal to the number of long and short products remaining at the end of the previous cycle. Thus, the number of short products containing the target sequence grow exponentially with each cycle. Preferably, PCR is carried out with a commercially available thermal cycler, e.g., Perkin Elmer model 9600 thermal cycler.

PCR amplification is carried out by contacting the sample with a composition containing first and second primers, sufficient quantities of the four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and dTTP) to effect the desired degree of sequence amplification, and a primer- and template dependent polynucleotide polymerizing agent, such as any enzyme capable of producing primer extension products, for example, E. coli DNA polymerase I, Klenow fragment of DNA polymerase I, T4 DNA polymerase, thermostable DNA polymerases isolated from Thermus aquaticus (Taq), which is available from a variety of sources (for example, Perkin Elmer), Thermus thermophilus (United States Biochemicals), Bacillus stereothermophilus (Bio-Rad), or Thermococcus litoralis ("Vent" polymerase, New England Biolabs), and the like.

An important feature of the invention is the selection of parameters in the amplification phase that results in well-resolved amplification products, e.g. as measured by well-resolved bands on an electrophoretic gel. The quality of the ligation products produced in the ligation phase are directly dependent on the quality of the amplification products. In this regard, an important parameter in PCR amplification is the annealing temperature employed. Preferably, the highest practical annealing is employed so that highly specific amplification is achieved and amplification of spurious targets is minimized.

After amplification, the temperature of the reaction mixture is lowered to implement OLA. The amount the temperature is lowered, of course, depends on the particular embodiment. Typically, the temperature is lowered from 20°C to 50°C to a second annealing temperature which facilitates specific annealing of the oligonucleotide probes to the target polynucleotide. That is, the second annealing temperature should be high enough to preclude the formation of duplexes having mismatches between oligonucleotide probes and the target polynucleotides. The OLA reaction for detecting mutations exploits the fact

that the ends of two single strands of DNA must be exactly aligned for DNA ligase to join them. If the terminal nucleotides of either end are not properly base-paired to the complementary strand, then the ligase cannot join them. Thus, for a chosen target oligonucleotide sequence, first and second oligonucleotide probes are prepared in which the terminal nucleotides are respectively complementary to the normal sequence and the mutant sequence.

Whenever PCR amplification is employed, an important feature of the invention is providing oligonucleotide probes with 3' termini which are incapable of being extended by DNA polymerases. This is accomplished in a variety of conventional ways. For probes having a 3' terminus which will not be ligated, blocking is conveniently effected by attaching a blocking group, e.g. a fluorescent dye, 3' phosphate, 3' amino, or like group, or by providing a probe having dideoxynucleotide at the 3' terminus. For probes having a 3' hydroxyl that will be ligated, the probe to which it will be ligated can be provided in a concentration to effectively displace any polymerase in the reaction mixture, thereby precluding extension.

In a preferred embodiment, one of the first or second oligonucleotide probes bears a fluorescent label such as 5-carboxyfluorescein (5-FAM), 6-carboxy-fluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), N,N,N',N'-tetramethyl-6-carboxy rhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4,7,2',4',5',7'-hexachloro-6-carboxy-fluorescein (HEX-1), 4,7,2',4',5',7'-hexachloro-5-carboxy-fluorescein (HEX-2), 2',4',5',7'-tetrachloro-5-carboxy-fluorescein (ZOE), 4,7,2',7'-tetrachloro-6-carboxy-fluorescein (TET-1), 1',2',7',8'-dibenzo-4,7-dichloro-5-carboxyfluorescein (NAN-2), and 1',2',7',8'-dibenzo-4,7-dichloro-6-carboxyfluorescein. In addition, ligation products may be detected by ELISA, sandwich-type nucleotide hybridization assays as described in U.S. Patent No. 4,868,105 to Urdea, or other methods which will be readily apparent to those of skill in the art. The first and second oligonucleotide probes are constructed to hybridize to adjacent nucleic acid sequences in a target polynucleotide. Thus, the orientation of the first oligonucleotide probes relative to the second oligonucleotide probes may be 5' to 3', as depicted in Figure 1, or 3' to 5'.

Preferably, oligonucleotide probes are fluorescently labeled by linking a fluorescent molecule to the non-ligating terminus of the probe. In order to facilitate detection in a multiplex assay, copies of different OLA reporter probes are labeled with different fluorescent labels. Guidance for selecting appropriate fluorescent labels can be found in Smith et al. (1987) Meth. Enzymol. 155:260-301, Karger et al. (1991) Nucl. Acids Res. 19:4955-4962, Haugland (1989) Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Inc., Eugene, OR). Preferred fluorescent labels include fluorescein and derivatives thereof, such as disclosed in U.S. Patent No. 4,318,846 to Khanna et al. and Lee et al. (1989) Cytometry 10:151-164, and 6-FAM, JOE, TAMRA, ROX, HEX-1, HEX-2, ZOE, TET-1 or NAN-2, as described above, and the like. Most preferably, when a plurality of fluorescent dyes are employed, they are spectrally resolvable, as taught by Fung, supra. As used herein, "spectrally resolvable" fluorescent dyes are those with quantum yields, emission bandwidths, and emission maxima that permit electrophoretically separated polynucleotides labeled therewith to be readily detected despite substantial overlap of the concentration bands of the separated polynucleotides.

In a preferred embodiment, the first oligonucleotide probes are complementary to variant nucleotide sequences which are 5' to the sequence to which the second oligonucleotide probe is complementary. Ligation occurs, if at all, between the 3' terminus of the first oligonucleotide probe and the 5' terminus of the second oligonucleotide probe. Therefore, in this embodiment, first oligonucleotide probes bear mobility modifiers, or detectable labels, on their 5' terminus. The 5' mobility modifiers, e.g., non-complementary nucleotide or nonnucleotide extensions are not affected by the 5' to 3' exonuclease activity of Taq polymerase because conditions are such that the extensions are not annealed during amplification. Also, they are present in sufficiently low concentrations to prevent appreciable exonuclease activity should annealing occur. In this preferred embodiment, detection may be achieved by coupling a detectable label to the 3' terminus of the second oligonucleotide probe. This allows detection of the ligated product and also acts to block 3' extension by Taq polymerase. Extension from the 3' end of the

first oligonucleotide probe may occur but it is not detected because it prevents ligation.

The reaction buffer used in the method of the invention must support the requirements of both the amplification scheme employed and OLA. Taq DNA ligase requires NAD+ as a cofactor, the divalent cation Mg2+ for activity, and its activity is stimulated by low concentrations of the monovalent cation K+ but not Na+ (Takahashi et al. (1984) J. Biol. Chem. 259:10041-10047). Optimal assay conditions for OLA reactions require 5 to 10 mM magnesium ions in the presence of 10 to 50 units of thermostable ligase. Thus, a reaction buffer which will find utility with the claimed coupled amplificationligation method is made up of, inter alia, 1 to 200 mM, preferably 25 to 50 mM, K+, 0.5 to 20 mM, preferably 1 to 5 mM Mg2+, and 0.5 to 20 mM, preferably 1 to 5 mM, NAD+.

Preferably, the method of the invention is carried out as an automated process which utilizes a thermostable enzyme. In this process the reaction mixture is cycled through PCR cycles, e.g., a denaturing region, a primer annealing region and a reaction region, and then through one or more OLA cycles. A machine may be employed which is specifically adapted for use with a thermostable enzyme, which utilizes temperature cycling without a liquid handling system, since the enzyme need not be added at every cycle.

As mentioned above, the invention includes kits for carrying out the method. Such kits include (a) a plurality of amplification primers, each amplification primer of the plurality being capable of annealing to one or more target polynucleotides at a first annealing temperature; (b) a plurality of oligonucleotide probes, each oligonucleotide probe being capable of annealing to the target polynucleotides at a second annealing temperature, such that substantially none of the oligonucleotide probes anneal to the target polynucleotide at the first annealing temperature; (c) means for amplifying the target polynucleotides using the plurality of amplification primers at a temperature greater than or equal to the first annealing temperature; and (d) means for ligating oligonucleotide probes at a temperature equal to or less than the second annealing temperature to form one or more ligation products. Preferably, kits of the invention further include instructions pertinent for the particular embodiment of the kit, such instructions describing the

oligonucleotide probes and amplification primers included and the appropriate first and second annealing temperatures for operation of the method. In the case of PCR amplification, kits further include a DNA polymerase, nucleoside triphosphates, a DNA ligase, and reaction buffer for the coupled ligation and amplification. Most preferably, oligonucleotide probes and amplification primer of the kit are selected from the sequences of Tables 1 and 2 for analyzing the CFTR locus.

Experimental

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the method of the invention, and are not intended to limit the scope of that which the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in °C and pressure is at or near atmospheric.

The detection and distinction of allelic variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by method of the invention is exemplified. This method can detect all types of single base substitution mutations in addition to small deletions and insertions. The technique involves an initial PCR amplification of small segments (individual exons or intronic fragments) of the CFTR gene, followed by an oligonucleotide ligation reaction which thereby allows the simultaneous screening for a number of mutations within each amplified region. In the OLA procedure two juxtaposed synthetic oligonucleotide probes hybridizing to a target DNA strand are enzymatically joined by thermostable DNA ligase only if there is correct base pairing at the junction region of the two hybridizing probes. To distinguish between two alternative DNA sequences, three oligonucleotide probes were used as shown in Figure 1. Normal and mutant diagnostic OLA probes were designed such that their 3' terminal base was homologous to either the normal or the altered base of a particular mutation under study. The allele-specific oligonucleotides were modified at their 5' termini by addition of different sized non-complementary tails to enable identification of different allelic products by size in polyacrylamide gels. The reporter oligonucleotide probe, designed to

hybridize immediately downstream of the two allelic or discriminating probes, was 5'-phosphorylated and modified by the addition of the fluorescent dye 5-FAM to its 3' end. Repeated thermocycling between the annealing temperature of the oligonucleotide probes, i.e., the second annealing temperature in this embodiment, and a denaturation temperature for the probes resulted in linear amplification of ligation products. The ligation products were then analyzed by electrophoresis on 8% denaturing polyacrylamide gels on the Applied Biosystems Model 373A DNA sequencer.

Human genomic DNA was prepared from peripheral blood nucleated cells and buccal cells. DNA was isolated from whole blood using the guanidinium method for extracting DNA (Chirgwin et al. (1979) Biochemistry 18:5294-5296; Chehab et al. (1992), supra). Briefly, 3 to 5 ml of stabilized whole blood (ethylene diamine tetraacetate (EDTA) or citrate) was mixed with 45 ml of lysis solution (0.32 M sucrose, 10 mM Tris HCl, pH 8.0, 5 mM MgCl2, 1% triton X-100) and nuclei were pelleted by centrifugation at 1500 rpm for 20 min. Nuclei were resuspended in 2 ml of guanidinium thiocyanate (5 M guanidine thiocyanate, 50 mM Tris HCl, pH 8.0, 10 mM EDTA), extracted by rotation for 15 min, and DNA was precipitated by addition of an equal volume of isopropyl alcohol. Purified DNA was dissolved in a small volume of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) or sterile water.

Cells from the mucosal surface of the buccal cavity were obtained by gently scraping with a brush or toothpick. After collection entrapped buccal cells were dislodged by gentle agitation into 500 μl of PBS in a microcentrifuge tube and pelleted by centrifugation at 1200 g for 5 min. DNA was either extracted from buccal cells as described above or by resuspending the cells in a 50 to 200 μl volume of sterile water and boiling for 20 min. Cell debris was removed by brief centrifugation, and 5 μl of the supernatant DNA solution was used in DNA amplification reactions. In some cases, samples were digested with proteinase K (100 μg/ml) for several hr at 50°C before boiling.

All oligonucleotides used were synthesized by an Applied Biosystems Model 394 DNA synthesizer (Foster City, CA) using standard cyanoethyl phosphoramidite chemistry (Giusti et al. (1993) PCR Methods Applic. 2:223-227). Reporter oligonucleotide probes were synthesized with 3' Amine-ON

CPG columns (5220-1, Clontech Laboratories, Inc., Palo Alto, CA) to derivatize the 3' end for subsequent labeling with the fluorescent dye, such as 5-carboxy-fluorescein (FAM), 2',7'dimethoxy-4',5'-dichloro-6-carboxy-fluorescein, N,N,N',N'-tetramethyl-6-carboxy rhodamine, 6-carboxyrhodamine X, or the like. The 5' end of each reporter oligonucleotide probe was phosphorylated using 5' Phosphate-ON (5210-1, Clontech Laboratories, Inc., Palo Alto, CA) to chemically phosphorylate the 5' terminus. Dye-labeled, phosphorylated oligonucleotides were purified from nonconjugated oligonucleotides by reverse-phase HPLC (Giusti et al., supra). Normal and mutant allelic oligonucleotide probes were purified using oligonucleotide purification cartridges (Applied Biosystems). Purified oligonucleotide probes were lyophilized, resuspended in sterile distilled water, and quantified spectrophotometrically. Sequences of primers and probes used in the Example provided below are depicted in Tables 1 and 2. Some primer and probe sequences are taken from Zielenski et al, Genomics, 10: 214-228 (1991).

Example 1

Analysis of the Fifteen Most Common Cystic Fibrosis Mutations with Coupled Amplification and Ligation

The method of performing a multiplex polymerase chain reaction (PCR) and ligase amplification reaction (OLA) uses PCR primers with high Tm's (76°C to 116°C), OLA oligonucleotides with Tm's between 52°C to 68°C, a two-step PCR cycle that employs a denaturation step done at 94°C and an annealing elongation step done at 72°C, and one to three two-step OLA cycles that have a denaturation step done at 94°C and a hybridization step done at between about 52°C to 56°C. Coupled amplification-ligation reactions were performed in a total volume of 50 μl in 0.2 ml thin-wall tubes in a Perkin-Elmer 9600 DNA thermocycler. Each reaction contained 2 μl of DNA (100-200 ng) extracted from peripheral blood or 2 μl of DNA from boiled mucosal cell lysates, primers for multiplex PCR (200-800 nM) of CFTR exons 10, 11, 20, 21 and intron 19, or CFTR exons 4, 11, 14b and 19, and oligonucleotide probes (2.5-12.5 nM) for CFTR mutations G542X, G551D, ΔF508, W1282X, N1303, 3905insT and 3849+10kbCT, or 3849+4AG, 3659delC, R117H, R1162X, 117-

1GT, 621+1GT R553X and 2789+5GA, respectively, in buffer containing 10 mM Tris HCl, pH 8.3, 50 mM KCl, 4.5 mM MgCl2, 1 mM NAD+, 200-600 μM each dATP, dCTP, dGTP and dTTP, 5 units cloned Taq DNA polymerase and 20 units of Thermus aquaticus DNA ligase (Barany et al. (1991), supra).

Following a 5 min denaturation at 94°C, samples were subjected to 25 PCR amplification cycles each consisting of 94°C for 30 sec and 72°C for 1.5 min. This was followed by a second denaturation at 98°C for 3 min and then 1-10 oligonucleotide ligation cycles of 94°C for 30 sec and 55°C for 3 min. Samples were stored, if at all, at -20°C following the method and prior to analysis.

Amplification-ligation products were analyzed by taking a 0.5-2.0 μl aliquot of each reaction mixture, 10 fmol of internal lane standard consisting of oligomers of 30 to 70 bases in size labeled with the dye ROX (6-carboxyrhodamine X) (Applied Biosystems) and 4 μl formamide loading buffer (deionized formamide:50 mM EDTA, 5:1 (v/v)). Samples were heat denatured at 100°C for 5 min, rapidly cooled on ice and loaded onto an 8% polyacrylamide denaturing sequencing gel. Gels were electrophoresed for 3 hr at 1500 V in an Applied Biosystem Model 373A fluorescent DNA sequencer. The location and relative quantity of ligation products were automatically recorded with Genescan 672 software (Applied Biosystem). PCR products were analyzed by electrophoresis in 3% MetaPhor agarose (FMC BioProducts, Rockland, ME) gels in 1x Tris-borate EDTA (TBE) buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 8.0) at 100 V for 5-6 hours and visualized by staining with 0.5 μg/ml ethidium bromide.

Figure 2 depicts the results of these assays. Figures 2A and 2B show the ability of the assay to accurately discriminate 7 and 8, respectively, of the 15 most common cystic fibrosis mutations.

Table 1. PCR Primers

Primer Sequence (5'->3')[a]	T_m (°C)[b]	Region Amplified[c]	Size (bp)[d]
F: GAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCT	81.6	Exon 3	213
R: CCTTTATATTTTTACACCTATTCACCAGATTTCGTAGTC	76.3		
F: AGAGTTTCAACATATGGTATGACCCTC	66.6	Exon 4	451
R: CCCTTACTTGTACCAGCTCACTACCTA	66.6		
F: ATTTCTGCCTAGATGCTGGGAAATAAAAC	70.6	Exon 5	402
R: CCAGGAAAACTCCGCCTTTCCAGTTG	70.3		
F: CTCTAGAGACCATGCTCAGATCTTCCAT	60.7	Exon 7	416
R: GCAAAGTTCATTAGAACTGATCTATTGACT	60.5		
F: TATACAGTGTAATGGATCATGGGCCATGT	62.0	Exon 9	570
R: GTGCAAGATACAGTGTTGAATGTGGTGCA	70.6		
F: GTGCATAGCAGAGTACCTGAAACAGGAAGTA	71.0	Exon 10	503
R: TGATCCATTCACAGTAGCTTACCCATAGAGG	67.7		
F: CAACTGTGGTTAAAGCAATAGTGTGATTATATGATTA	67.0	Exon 11	425
R: GCACAGATTCTGAGTAACCATAATCTCTACCAAATC	67.7		
F: GTGAATCGATGTGGTGACCATATTGTAATGCATGTA	67.7	Exon 12	339
R: ACCATGCTACATTCTGCCATACCAACAATGGTGAAC	63.6		
F: CTCATGGGATGTGATTCTTTCGACCAATTTAGTG	75.9	Exon 13	297
R: AGAATCTGGTACTAAGGACAGCCTTCTCTCTAA	74.0		
F: CATCACAAATAATAGTACTTAGAACACCTAGTACAGCTGCT	76.4	Exon 14b	476
R: GCCCTGAACTCCTGGGCTCAAGTGATCCTCCTGC	70.1		
F: AATTATAATCACCTTGTGGATCTAAATTTCAGTTGACTTGTC	70.1	Intron 19	300
R: TTTAAGACATACCCTAAATCTAAGTCAGTGTTTTCTAATAAC	76.4		
F: GCCCGACAAATAAGCAAGTGACAAATAG	73.9	Exon 19	454
R: GCTAACACATTGCTTCAGGCTACTGGG	75.0		
F: GGTCAGGATTGAAAGTGTGCAACAAGGTTTGAATGAATAAG	64.7	Exon 20	473
R: CTATGAGAAAACTGCACTGGAGAAAAAAAAGAGAGCAATG	62.7		
F: AATGTTCACAAGGGACTCCAAATATTGCTGTAGTATTTG	60.2	Exon 21	403
R: TCCAGTCAAAAGTACCTGTTGCTCCAGGTATGTTAGGGTA	63.7		

[a] Primer sequences indicated in bold text are from Zielenski et al.

[b] T_m values were calculated by nearest neighbor analysis.

[c] Region of the CFTR gene amplified.

[d] PCR product size in base pairs.

Table 2 Oligonucleotide Probes for Detection of CF Mutations

Mutation	Wild-type Probe (5'-3')[a]	Mutant Probe (5'-3')[a]	Common Probe (5'-3')[b]	Ligation Product Size (bases)	
				Wild-type	Mutant
ΔF508	(a_{21}) CACCATTAAAGAAAATATCATCTT	(a_{20})AGCACCATTAAAGAAAATATCAT	TGATGATTTCCTTATGATGAAATAT	67	65
	(a_{31})- CACCATTAAAGAAAATATCATCTT	(a_{20})-AGCACCATTAAAGAAAATATCAT		48	47
G542X	CTTGATTCCACCTTCTCC	GTGTGATTCCACCTTCTCA			
G551D	(a_{21})-TAAAGAAATTCTTGCTCGATTGAG	TAAAGAAATTCTTGCTCGATTGAT	AAGAACTATATTATGTCTTCTCT	39	41
W1282X	(a_{31})-TATCACTCCAAAGGCTTCCTC	(a_{21})-TATCACTCCAAAGGCTTCCTT	CTCCACTCAGTGTGATTCCA	45	43
N1303K	(c_{41})-TATTTTTTCTGGAACATTTAGAAA AAAG	(c_{41})-TATTTTTTCTGGAACATTTAGAAA AAAC	CACTGTTGCAAAGTTATTGAATCC	51	53
3005insT	(a_{10})-AAGAGTACTTTGTTATCAGCTTTTTT	(a_{12})-AAGAGTACTTTGTTATCAGCTTTTTTT	TTGGATCCCTATGAACACTGAAG	65	67
3849+10kbC->T	(a_{25}) ATCTGTTGCAGTAATAAAATGGC	(a_{20})-CATCTGTTGCAGTAATAAAATGGT	GAGACTACTGAACACTGAAGAAG	58	62
3849+4A->G	(a) CCTGTCCAGAGGTGA	CTGTCCCAGAGGTGG	CTATGAAGACACCCTGAAAGGAA	70	72
3659delC	(a_2) CAACAAAGATAAACCTAC	CCAACAAAGATAAACCTA	GATTTGAACACTGCTTGCT	38	34
I1171I	ACTAATAAAICCCATAAAIG	(a_{21})-ACTAGATAAATCGCAATAGAGT	CAATTGAAGCAAACCATACA	41	39
R1162X	(a)-TTTCAGATGCAATCTGTGAGCT	(a_{21})-TTTCAGATGCAATCTGTGAGCT	GTTCCTCCTTGATTATCCGGGT	43	45
1717-1G->A	(a_{41}) TCTGCAAACTTGGAGATGTCC	(a_{41})-TCTGCAAACTTGGAGATGTGT	GAGTCTTTAAGTTCATTGACATGG	47	49
621+1G->T	(a_{31})-TATGTTTAGTTTGATTTATAAGAGG	(a_{41})-TATGTTTAGTTTGATTTATAAGAGT	TATTACCAAAAATAGAAAATTAGAGA	61	63
R553X	(a_{10}) TGCTAAAGAAATTCTTGCTCA	(a_{20})-TGCTAAAGAAATTCTTGCTCA	TAATACTTCCTGCACAGGCCC	65	67
2789+5G->A	(c_{21}) CACAATAGGACATGGAATAT	(c_{25}) CACAATAGGACATGGAATAA	TGACCTCCAGTCAGTATGTGA	58	6
			TCACTTTCCAAGGAAGCCAG	68	64

[a]5'-Poly(t) or poly(c) extensions were added to wild-type and mutant probes for multiplex detection of alleles by gel electrophoresis.

[b]Probes were 5'-phosphorylated and fluorescently labeled at their 3'-ends with the fluorescein dye FAM.

RECTIFIED SHEET (RULE 91)
ISA/EP

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Perkin-Elmer Corporation, Applied Biosystems Division

(ii) TITLE OF INVENTION: Coupled Amplification and Ligation Method

(iii) NUMBER OF SEQUENCES: 58

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: David J. Weitz, Haynes & Davis

(B) STREET: 2180 Sand Hill Road, Suite 310

(C) CITY: Menlo Park

(D) STATE: California

(E) COUNTRY: USA

(F) ZIP: 94025-6935

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch diskette

(B) COMPUTER: IBM compatible

(C) OPERATING SYSTEM: Microsoft Windows 3.1/DOS 5.0

(D) SOFTWARE: Wordperfect for windows 6.0,
ASCII (DOS) TEXT format

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/292,686

(B) FILING DATE: 19-AUG-94

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: David J. Weitz

(B) REGISTRATION NUMBER: 38,362

(C) REFERENCE/DOCKET NUMBER: PELM4215WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 233-0188
(B) TELEFAX: (415) 233-1129

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
AGAGTTTCAA CATATGGTAT GACCCTC 27

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CCCTTACTTG TACCAGCTCA CTACCTA 27

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
GTGCATAGCA GAGTACCCGA AACAFFAAGT A 31

(2) INFORMATION FOR SEQ ID NO: 4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

TGATCCATTC ACAGTAGCTT ACCCATAGAG G 31

(2) INFORMATION FOR SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

CAACTGTGGT TAAAGCAATA GTGTGATTAT ATGATTA 37

(2) INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

GCACAGATTC TGAGTAACCA TAATCTCTAC CAAATC 36

(2) INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

CATCACAAAT AATAGTACTT AGAACACCTA GTACAGCTGC T 41

(2) INFORMATION FOR SEQ ID NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

GCCCTGAACT CCTGGGCTCA AGTGATCCTC CTGC 34

(2) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

AATTATAATC ACCTTGTGGA TCTAAATTTC AGTTGACTTG TC 42

(2) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

TTTAAGACAT ACCCTAAATC TAAGTCAGTG TTTTCTAATA AC 42

(2) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

GCCCGACAAA TAACCAAGTG ACAAATAG 28

(2) INFORMATION FOR SEQ ID NO: 12

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

GCTAACACAT TGCTTCAGGC TACTGG 26

(2) INFORMATION FOR SEQ ID NO: 13

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

GGTCAGGATT GAAAGTGTGC AACAAGGTTT GAATGAATAA G 41

(2) INFORMATION FOR SEQ ID NO: 14

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

CTATGAGAAA ACTGCACTGG AGAAAAAAAA GACAGCAATG 40

(2) INFORMATION FOR SEQ ID NO: 15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15
AATGTTCACA AGGGACTCCA AATATTGCYG AGTATTTG 39

(2) INFORMATION FOR SEQ ID NO: 16
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16
TCCAGTCAAA AGTACCTGTT GCTCCAGGTA TGTTAGGGTA 40

(2) INFORMATION FOR SEQ ID NO: 17
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17
AAAAAAAAAA AAAAAAAAAA GGCACCATTA AAGAAAATAT CAT 43

(2) INFORMATION FOR SEQ ID NO: 18
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18

AAGGCACCAT TAAAGAAAAT ATCAT 25

(2) INFORMATION FOR SEQ ID NO: 19

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19

GTGTGATTCC ACCTTCTCA 19

(2) INFORMATION FOR SEQ ID NO: 20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

TAAAGAAATT CTTGCTCGTT GAT 23

(2) INFORMATION FOR SEQ ID NO: 21

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21

AAAAAAATAT CACTCCAAAG GCTTTCCTT 29

(2) INFORMATION FOR SEQ ID NO: 22

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22

CCCCCCTATT TTTTCTGGAA CATTTAGAAA AAAG 34

(2) INFORMATION FOR SEQ ID NO: 23

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23

AAAAAAAAAA AAAAGAGTAC TTTGTTATCA GCTTTTTTT 39

(2) INFORMATION FOR SEQ ID NO: 24

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24

AAAAAAAAAA AAAAAAAAAA AAAAAACATC TGTTGCAGTA

ATAAAATGGT 50

(2) INFORMATION FOR SEQ ID NO: 25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25

CTGGCCAGAG GGTGG 15

(2) INFORMATION FOR SEQ ID NO: 26

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26

CCAACAGAAG GTAAACCTA 19

(2) INFORMATION FOR SEQ ID NO: 27

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27

AAACTAGATA AATCGCGATA GAGT 24

(2) INFORMATION FOR SEQ ID NO: 28

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28

AAATTTCAGA TGCGATCTGT GAGCT 25

(2) INFORMATION FOR SEQ ID NO: 29

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29

AAAAAATCTG CAAACTTGGA GATGTCT 27

(2) INFORMATION FOR SEQ ID NO: 30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30

AAAAAAAAAT ATGTTTAGTT TGATTTATAA GAAGT 35

(2) INFORMATION FOR SEQ ID NO: 31

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31

AAAAAAAAAA AAAAAAAAAA TTGCTAAAGA AATTCTTGCT CA 42

(2) INFORMATION FOR SEQ ID NO: 32

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32

CCCCCCCCCC CCCCCCCCCC CCCCCCACAA TAGGACATGG AATAT 45

(2) INFORMATION FOR SEQ ID NO: 33

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33

AAAAAAAAAA AAAAAAAAAA ACACCATTAA AGAAAATATC ATCTT 45

(2) INFORMATION FOR SEQ ID NO: 34

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34

AAACACCATT AAAGAAAATA TCATCTT 27

(2) INFORMATION FOR SEQ ID NO: 35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35

GTGATTCCAC CTTCTCC 17

(2) INFORMATION FOR SEQ ID NO: 36

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36

AATAAAGAAA TTCTTGCTCG TTGAC 25

(2) INFORMATION FOR SEQ ID NO: 37

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37

AAAAATATCA CTCCAAAGGC TTTCCTC 27

(2) INFORMATION FOR SEQ ID NO: 38

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38

CCCCTATTTT TTCTGGAACA TTTAGAAAAA AC 32

(2) INFORMATION FOR SEQ ID NO: 39

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39

AAAAAAAAAA AAGAGTACTT TGTTATCAGO TTTTTT 36

(2) INFORMATION FOR SEQ ID NO: 40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40

AAAAAAAAAA AAAAAAAAAA AAAAAATCTG TTGCAGTAAT AAAATGGC 48

(2) INFORMATION FOR SEQ ID NO: 41

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41

ACCTGGCCAG AGGGTGA 17

(2) INFORMATION FOR SEQ ID NO: 42

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42

AACAACAGAA GGTAAACCTA C 21

(2) INFORMATION FOR SEQ ID NO: 43

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43

ACTAGATAAA TCGCGATAGA GC 22

(2) INFORMATION FOR SEQ ID NO: 44

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44

ATTTCAGATG CGATCTGTGA GCC 23

(2) INFORMATION FOR SEQ ID NO: 45

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45

AAAATCTGCA AACTTGGAGA TGTCC 25

(2) INFORMATION FOR SEQ ID NO: 46

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46

AAAAAAATAT GTTTAGTTTG ATTTATAAGA AGG 33

(2) INFORMATION FOR SEQ ID NO: 47

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47

AAAAAAAAAA AAAAAAAATG CTAAAGAAAT TCTTGCTCG 39

(2) INFORMATION FOR SEQ ID NO: 48

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48

CCCCCCCCCC CCCCCCCCCC CCCCCCCCAC AATAGGACAT GGAATAC 47

(2) INFORMATION FOR SEQ ID NO: 49

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49

ATTTCTGCCT AGATGCTGGG AAATAAAAC 29

(2) INFORMATION FOR SEQ ID NO: 50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50

CCAGGAAAAC TCCGCCTTTC CAGTTG 26

(2) INFORMATION FOR SEQ ID NO: 51

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51

CTCTAGAGAC CATGCTCAGA TCTTCCAT 28

(2) INFORMATION FOR SEQ ID NO: 52

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52

GCAAAGTTCA TTAGAACTGA TCTATTGACT 30

(2) INFORMATION FOR SEQ ID NO: 53

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53

TATACAGTGT ATGGATCATG GGCCATGT 28

(2) INFORMATION FOR SEQ ID NO: 54

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54

GTGCAAGATA CAGTGTTGAA TGTGGTGCA 29

(2) INFORMATION FOR SEQ ID NO: 55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55

GTGAATCGAT GTGGTGACCA TATTTAATGC ATGTA 35

(2) INFORMATION FOR SEQ ID NO: 56

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56

ACCATGCTAC ATTCTGCCAT ACCAACAATG GTGAAC 36

(2) INFORMATION FOR SEQ ID NO: 57

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57

CTCATGGGAT GTGATTCTTT CGACCAATTT AGTG 34

(2) INFORMATION FOR SEQ ID NO: 58

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58

AGAATCTGGT ACTAAGGACA GCCTTCTCTC TAA 33

CLAIMS

1. A method for detecting one or more polynucleotides in a sample, the method comprising the steps of:

(a) providing a plurality of amplification primers, each amplification primer of the plurality being capable of annealing to one or more target polynucleotides at a first annealing temperature;

(b) providing a plurality of oligonucleotide probes, each oligonucleotide probe of the plurality being capable of annealing to the target polynucleotides at a second annealing temperature, such that substantially none of the oligonucleotide probes anneal to the target polynucleotide at the first annealing temperature;

(c) amplifying the target polynucleotides using the plurality of amplification primers at a temperature greater than or equal to the first annealing temperature;

(d) ligating oligonucleotide probes of the plurality that specifically hybridize to the one or more target polynucleotides at a temperature equal to or less than the second annealing temperature to form one or more ligation products; and

(e) detecting the one or more ligation products.

2. The method of claim 1 wherein said step of amplifying includes amplifying by polymerase chain reaction or ligase chain reaction.

3. The method of claim 2 wherein said first annealing temperature is between about 72°C and about 84°C said second annealing temperature is between about 30°C and about 55°C.

4. The method of claim 3 wherein said first annealing temperature is between about 72°C and about 75°C said second annealing temperature is between about 40°C and about 55°C.

5. The method of claim 1 wherein said ligation products have distinct electrophoretic mobilities and said step of detecting includes separating said ligation products by electrophoresis.

6. The method of claim 1 wherein said ligation products have distinct fluorescent labels and said step of detecting includes measuring a fluorescent signal generated by the distinct fluorescent labels.

7. The method of claim 6 wherein said ligation products have distinct electrophoretic mobilities and said step of detecting includes separating said ligation products by electrophoresis.

8. The method of claim 7 wherein said fluorescent labels are selected from the group consisting of 5-carboxyfluorescein, 6-carboxyfluorescein, 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein, N,N,N',N'-tetramethyl-6-carboxy rhodamine, 6-carboxyrhodamine X, 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein, 4,7,2',4',5',7'-hexachloro-5-carboxyfluorescein, 2',4',5',7'-tetrachloro-5-carboxyfluorescein, 4,7,2',7'-tetrachloro-6-carboxy-fluorescein, 1',2',7',8'-dibenzo-4,7-dichloro-5-carboxyfluorescein, and 1',2',7',8'-dibenzo-4,7-dichloro-6-carboxyfluorescein.

9. The method of claim 8 wherein said one or more target polynucleotides are alleles or mutations of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR).

10. The method of claim 9 wherein said one or more target polynucleotides are alleles or mutations of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) selected from the group consisting of ΔF508, G542X, G551D, W1282X, N1303K 3905insT, 3849+10kbCT, 3849+4AG, 3659delC, R117H, R1162X, 1717-1GA, 621+1GT, R553X, 2789+5GA, R347P, 2184delA, 1078delT, R334W, 711+1GT, G85E, 1898+1GA, A455E, S549R, S549N, R560T, ΔI507, Q493X, V520F and Y122X.

11. A kit for detecting one or more polynucleotides in a sample, the kit comprising:

(a) a plurality of amplification primers, each amplification primer of the plurality being capable of annealing to one or more target polynucleotides at a first annealing temperature;

(b) a plurality of oligonucleotide probes, each oligonucleotide probe being capable of annealing to the target polynucleotides at a second annealing temperature, such that substantially none of the oligonucleotide probes anneal to the target polynucleotide at the first annealing temperature;

(c) means for amplifying the target polynucleotides using the plurality of amplification primers at a temperature greater than or equal to the first annealing temperature; and

(d) means for ligating oligonucleotide probes at a temperature equal to or less than the second annealing temperature to form one or more ligation products.

12. The kit of claim 11 further comprising:

(a) instructions for carrying out the method of claim 1;

(b) a DNA polymerase;

(c) nucleoside triphosphates;

(d) a DNA ligase; and

(e) a reaction buffer for coupled amplification and ligation.

13. The kit of claim 12 wherein said oligonucleotide probes and said amplification primers are capable of detecting alleles or mutations of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) selected from the group consisting of ΔF508, G542X, G551D, W1282X, N1303K 3905insT, 3849+10kbCT, 3849+4AG, 3659delC, R117H, R1162X, 1717-1GA, 621+1GT, R553X, 2789+5GA, R347P, 2184delA, 1078delT, R334W, 711+1GT, G85E, 1898+1GA, A455E, S549R, S549N, R560T, Δ1507, Q493X, V520F and Y122X.

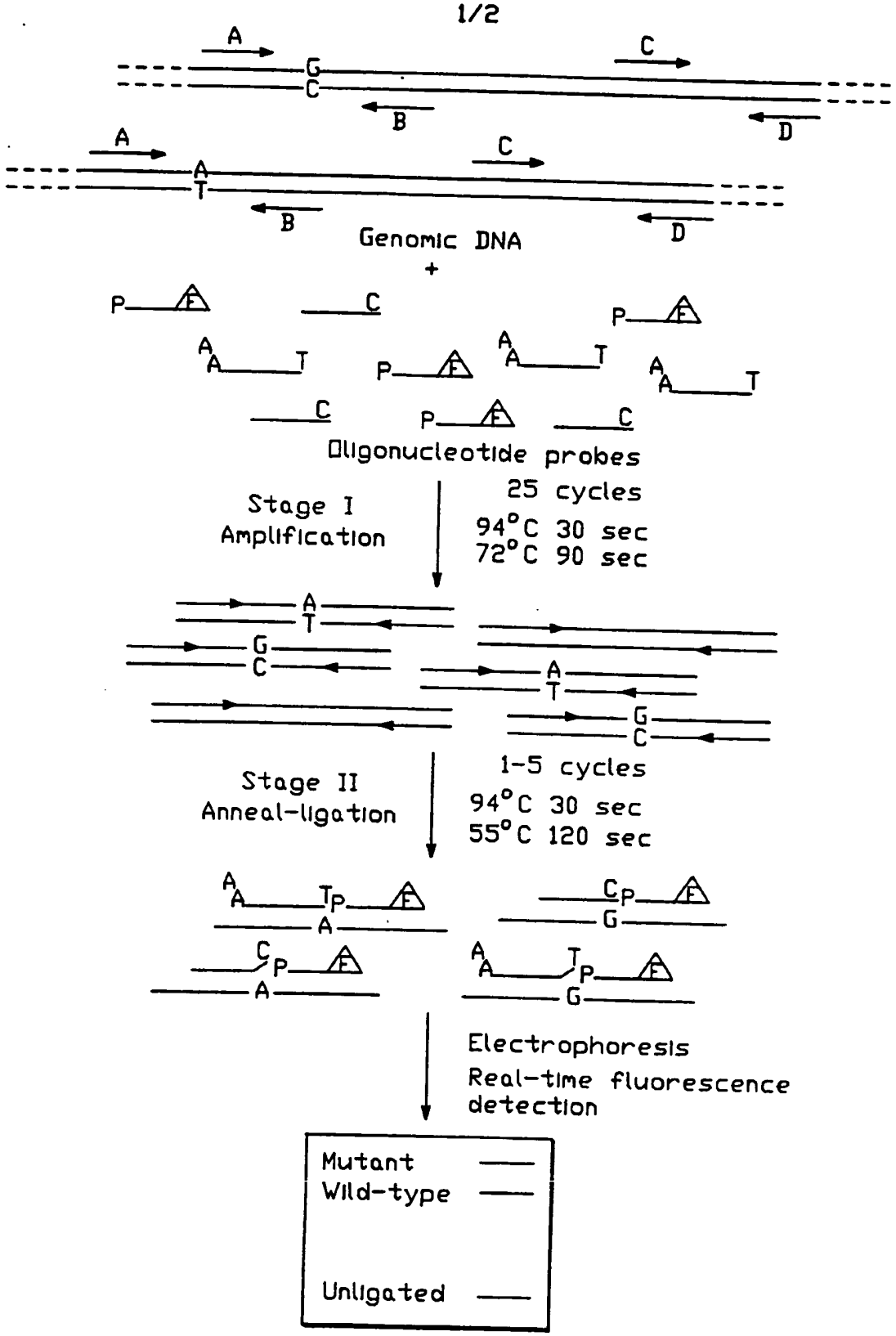

A
C
G
C
B
D
A
C
A
T
B
D
Genomic DNA
+
P
F
C
A
A
T
Oligonucleotide probes
Stage I
Amplification
25 cycles
94°C 30 sec
72°C 90 sec
Stage II
Anneal-ligation
1-5 cycles
94°C 30 sec
55°C 120 sec
Electrophoresis
Real-time fluorescence
detection
Mutant
Wild-type
Unligated

FIG. 1

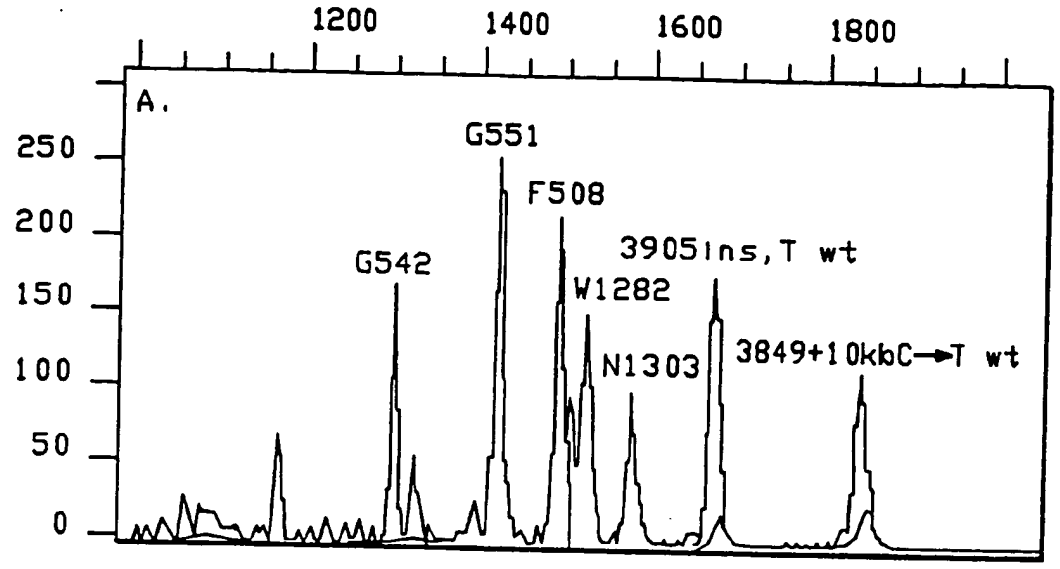

1200
1400
1600
1800
A.
250
200
150
100
50
0
G551
F508
G542
3905ins,T wt
W1282
N1303
3849+10kbC→T wt

FIG.2A

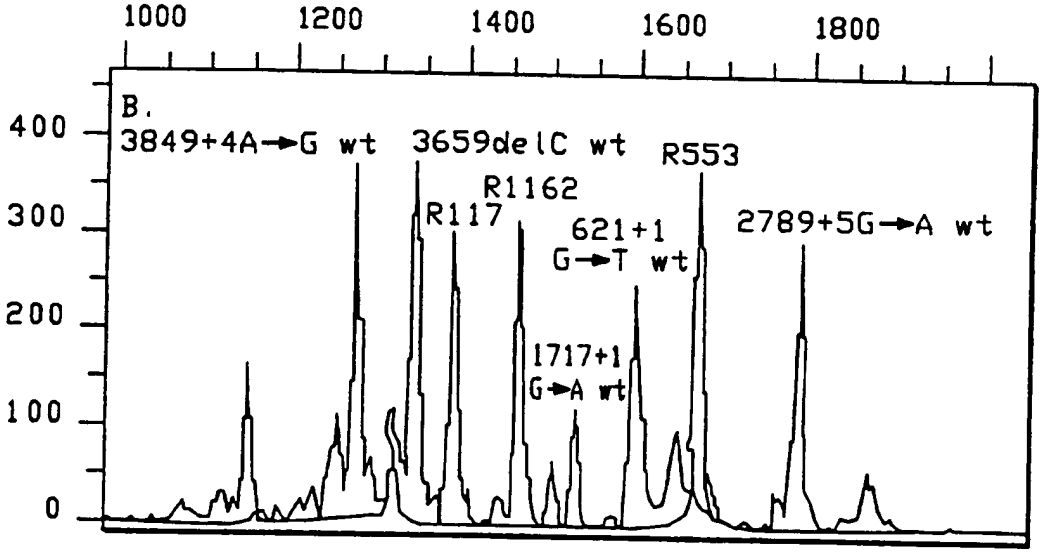

1000
1200
1400
1600
1800
B.
400
300
200
100
0
3849+4A→G wt
3659delC wt
R553
R1162
R117
621+1
G→T wt
2789+5G→A wt
1717+1
G→A wt

FIG.2B